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Despite the importance of *BRCA1* and *BRCA2* genes in breast cancer suppression, little concrete information is available concerning the functions of their encoded products. Recent studies have indicated a physical interaction of BRCA1 with the Rad50/Mre11/NBS1 complex and of BRCA2 with Rad51, which are key components of the *RAD52* protein group that mediate homologous recombination and recombinational DNA repair. These findings strongly suggest that both BRCA1 and BRCA2 proteins have a function in recombination and DNA repair. For defining the mechanism of the recombination machinery and the roles of BRCA1 and BRCA2 proteins in recombination, we have (i) purified from nuclear extracts of Raji cells the Rad50/Mre11/NBS1 complex and have carried out characterization of the nuclease activities of this complex, (ii) purified the Rad51 protein and established in vitro systems for examining the biochemical properties of this recombinase, and (iii) expressed BRCA2 domains that are known to interact with Rad51 protein. A variety of studies will be carried out to delineate the mechanism of the recombination machinery and to examine the functional and physical interactions among components of the recombination machinery, BRCA1, and BRCA2.

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____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Patrick M. Sung June 25 1994

PI - Signature Date

ANNUAL SUMMARY

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INTRODUCTION

Recent studies on the tumor suppressor genes *BRCA1*, *BRCA2*, *ATM*, and *NBS1* indicate that they affect DNA double-strand break (DSB) repair by homologous recombination, being mediated by a group of proteins known as the *RAD52* epistasis group. As the first step toward delineating the manner in which these tumor suppressor proteins modulate DSB repair in human cells and prevent cancer development, including breast cancers, the mechanism of the DSB repair machinery is being dissected. Existing evidence suggests that during DSB repair, the end of the break is processed by a multi-subunit nuclease to create a single-stranded DNA region. The binding of a number of recombination proteins to this single-stranded DNA results in the formation of a nucleoprotein complex, which subsequently conducts a search for a DNA homolog and mediates the formation of hybrid or heteroduplex DNA with the homolog. Later in the repair process, DNA synthesis and DNA ligation are required to complete the reaction. To achieve our research objectives, molecular studies are being carried out in our laboratory to examine the mechanisms of DSB end-processing and heteroduplex DNA formation, and to study the role of BRCA1, BRCA2, and various other tumor suppressors in these reactions.

BODY

Purification of a protein complex consisting of Rad50, Mre11, and NBS1 proteins

The Rad50, Mre11, and NBS1 proteins were identified in the nuclear extract of human Burkitt's lymphoma cells (Raji cells; purchased from the National Cell Culture Center in Minneapolis) by immunoblotting with mouse polyclonal sera raised against GST-Rad50, GST-Mre11, and GST-NBS1. A single protein species was detected with all three anti-sera in nuclear extract, and the sizes of these immunoreactive proteins are in excellent agreement with those predicted for the Rad50 (M.W. 153 kDa), Mre11 (M.W. 81 kDa), and NBS1 (95 kDa). Greater than 75% of the total cellular Rad50, Mre11, and NBS1 proteins were present in the nuclear extract, which was selected as starting material for the purification of the Mre11/Rad50/NBS1 complex. The clarified nuclear extract (Fraction I) from 80 ml of Raji cell pellet (from 50 L culture) was treated with ammonium sulfate to precipitate the Mre11/Rad50/NBS1 complex, which was redissolved in buffer and then subject to chromatographic fractionation in columns of Source Q, Hydroxyapatite, Sepharose-6B, Phenyl-Superose, Sepharose-6B, and Mini S (Trujillo et al, 1998). As determined by immunoblotting, Rad50, Mre11, and NBS1 proteins coeluted throughout purification. The protein pool from the last step of purification in Mini S (FractionVIII) was concentrated and stored in small portions at -70°C. When the Mini S pool (Fraction VIII) was analyzed by SDS-PAGE followed by staining with Coomassie Blue, only the Rad50, Mre11 and NBS1 protein bands were visible.

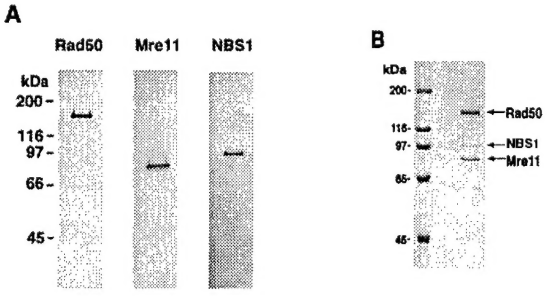

Figure 1. A. Purified Mre11/Rad50/NBS1 complex was subjected to immunblot analyses with anti-Rad50, anti-Mre11, and anti-NBS1 antisera. Each of the lane contains 100 ng of the purified protein complex. **B.** One μg of the Mre11/Rad50/NBS1 complex from the final step purification in Mini S was run in a 7% denaturing polyacrylamide gel followed by staining with Coomassie Blue (lane 2).

The Mre11/Rad50/NBS1 complex has a ssDNA endonuclease activity – We examined the purified Mre11/Rad50/NBS1 complex for a ssDNA endonuclease activity. To do this, ϕX174 circular ssDNA was incubated with Mre11/Rad50/NBS1 complex from the Mini S step in reaction buffer (30 mM KMOPS, pH 7.2, 1 mM DTT, 25 mM KCl, 1 mM ATP) containing 2 mM Mn^{2+}. The reaction was terminated after various time points by adding SDS to a final concentration of 0.3%. The reaction samples were run on a 0.8% agarose gel, and the DNA species were visualized by staining with ethidium bromide. As shown in Figure 2A, with increasing reaction time, the input ssDNA was converted to forms with progressively faster gel mobility, indicating that the Mre11/Rad50/NBS1 complex has a ssDNA endonuclease activity. Addition of SDS to 0.3 % at the beginning of the reaction abolished the nuclease activity (Figure 2B). Interestingly, the nuclease activity is dependent on manganese, which cannot be substituted by magnesium (Figure 2B).

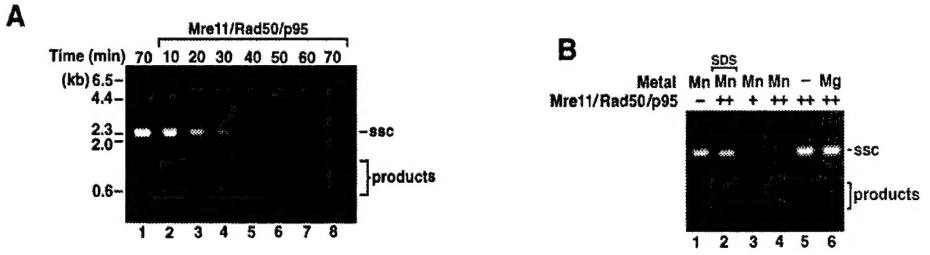

Figure 2. A. ϕX circular ssDNA, 1μg, was incubated with 1.5 μg of Fraction VIII Mre11/Rad50/NBS1 complex in 100 μl of reaction buffer. At the times indicated (lanes 2 to 8), 10μl of the reaction mixture was withdrawn, mixed with SDS, and then run on an 0.8% agarose gel, followed by staining with eithidium bromide to visualize the DNA species. In lane 1, the DNA was incubated in buffer without nuclease. **B.** ϕX circular ssDNA, 100 ng, was incubated with 100 ng (lane 3) and 200 ng (lanes 2, 4, 5, and 6) of Fraction VIII Mre11/Rad50/NBS1 complex in 10μl reaction buffer for 60 min. Mn^{2+} was omitted in the reaction in lane 5, and 2 mM Mg^{2+} was used instead of Mn^{2+} in lane 6. In lane 2, SDS (0.3%) was added at the beginning of the reaction. In lane 1, the DNA was incubated in buffer without nuclease. Symbols: ssc, circular ϕX ssDNA; products, the products formed as a result of Mre11/Rad50/NBS1 nucleolytic action.

Nuclease activity is specific for ssDNA - To examine whether the Mre11/Rad50/NBS1 nuclease will also act on dsDNA, we repeated the nuclease reaction, substituting the ϕX ssDNA with the double-stranded supercoiled form of ϕX DNA. As shown in Figure 3, Mre11/Rad50/NBS1 converted the supercoiled DNA (sc) into the nicked circular form (nc), but the incision of the supercoiled DNA clearly occurs at a much slower rate than the incision of ssDNA (see Figure 3). For instance, at the reaction end point of 70 min, only 14% of the supercoiled DNA had been incised, as compared to greater than 75% incision of the circular ssDNA by about 30 min (Figure 3A). The dsDNA endonuclease activity is also specifically dependent on manganese ion and is abolished by adding 0.3 % SDS at the beginning of the reaction (not shown). The activity of Mre11/Rad50/NBS1 is enhanced by increasing negative superhelicity in the substrate, indicating that the endonuclease acts on unwound regions in the DNA (not shown).

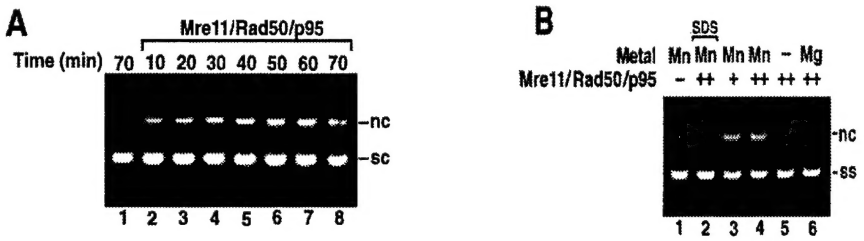

Figure 3. A. ϕX supercoiled DNA, 1 μg, was incubated with 1.5 μg of Faction VIII Mre11/Rad50/NBS1 complex in 100 μl of reaction buffer. At the times indicated (lanes 2 to 8), 10μl of the reaction mixture was withdrawn, mixed with SDS, and then run on an 0.8% agarose gel, followed by staining with eithidium bromide to visualize the DNA species. In lane 1, the DNA was incubated in buffer without nuclease. **B.** ϕX supercoiled DNA, 100 ng, was incubated with 75 ng (lane 3) and 150 ng (lanes 2, 4, 5, and 6) of Fraction VIII Mre11/Rad50/NBS1 complex in 10μl reaction buffer. Mn^{2+} was omitted in the reaction in lane 5, and 2 mM Mg^{2+} was used instead of Mn^{2+} in lane 6. In lane 2, SDS (0.3%) was added at the beginning of the reaction. In lane 1, the DNA was incubated in buffer without nuclease. Symbols: sc, supercoiled form; nc, nicked circular form.

Mre11/Rad50/NBS1 complex has a 3′ to 5′ exonclease activity – To examine whether the Mre11/Rad50/NBS1 complex has exonuclease activity, the purified complex was incubated with restriction DNA fragment that was labeled with ^{32}P either at the 3′ or the 5′ end, and the reaction mixtures analyzed by autoradiography following electrophoresis in agarose gels. The results from these experiments indicated that the Mre11 associated complex has a 3′ to 5′ exonuclease activity (Fig. 4); the exonuclease activity also shows a specific dependence on manganese (not shown). In other experiments that used short duplexes obtained by hybridizing 5′ end-labeled oligonucleotides, we have observed the removal of nucleotides from the 3′ of the DNA by the Mre11/Rad50/NBS1 complex. The products of exonuclease action are primarily mononucleotides (not shown). Unlike the ssDNA endonuclease activity, the exonuclease activity does not appear to be affected by ATP, and little if any exonuclease activity has been detected on unhybridized oligonucleotides, suggesting that the exonucleolytic function is specific for dsDNA (not shown).

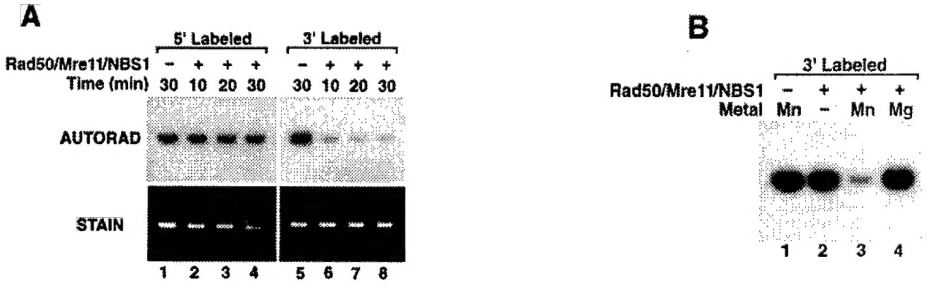

Figure 4. Mre11/Rad50/NBS1 complex has 3' to 5' exonuclease activity. **A.** 5' and 3' end-labeled DNA species were incubated Mre11/Rad50/NBS1 complex. At the times indicated, a portion of the reaction mixture was withdrawn and mixed with an equal volume of loading buffer containing 1 % SDS. The reaction samples were run in a 0.8% agarose gel, which was stained with eithidium bromide and photographed (lower panel), and then dried and

subjected to autoradiography (upper panel). In lanes 1 and 5, the end-labeled DNAs were incubated in buffer without Rad50-Mre11-p95. **B.** The 3' end-labeled DNA was incubated in buffer alone (lane 1) and with the Mre11/Rad50/NBS1 complex either without metal ion (lane 2), with 2 mM Mn^{2+} (Mn, lane 3), or with 2 mM Mg^{2+} (Mg, lane 4) as indicated.

Conclusions

There is a compelling body of evidence indicating that the recombinational repair of DNA double-strand breaks is modulated through the interactions of components of the repair machinery with a group of tumor suppressor proteins including ATM, BRCA1, and BRCA2. **Our main goal is to delineate the mechanism of DSB repair and the manner in which tumor suppressor proteins regulate the repair process.** To achieve this goal, a variety of biochemical studies are being carried out. We expect that information garnered from our studies will not only elucidate the mechanism of DSB repair and its regulation, but will also provide a much needed knowledge base for devising new strategies for the prevention and effective treatment of breast cancers.

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Appended Information

1. Key Research Accomplishments to date:

(i) Established procedure for purifying the complex of Rad50/Mre11/NBS1 from nuclear extracts of Raji cells.

(ii) Demonstration of nuclease activities in the Rad50/Mre11/NBS1 complex.

(iii) Construction of plasmids expressing different portions of BRCA2 for biochemical purification and characterization.

2. Reportable Outcomes

(i) Presentations:

1998 Invited Speaker, "Mechanism of Heteroduplex DNA Formation in Recombination and DNA Repair". Trinity University, San Antonio, Texas.

1999 Keystone Symposium on "Molecular Mechanisms in DNA Replication and Recombination", Taos, New Mexico. Title of presentation "Mechanism of Heteroduplex DNA Formation".

1999 MD Anderson Cancer Center at Smithville. "Enzymology of Recombination and DNA Double-strand Break Repair".

(ii) Funding applied for based on work supported by grant: DNA Repair and Tumor Supressor Genes (NCI PO1 Program Project (PI: Eva Lee) Leader of Unit 1: Initiation of Recombinational Repair; Estimated total direct and indirect costs for my unit: $1,108,389)